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Characterization and Diversity of Rhizobia Nodulating Prominent Arid Legumes of Thar Desert of India

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A B S T R A C T

A study was conducted to assess the characteristics and diversity existing in rhizobia nodulating leguminous trees (*Acacia senegal* and *Prosopis cineraria*) and crops (*Cyamopsis tetragonoloba* or guar and *Vigna aconitifolia* or moth bean) of arid zone of India. Sixty eight rhizobial cultures isolated from root nodules were characterized at phenotypic and molecular level. Sixty percent of the isolates were classified as fast or very fast growers and 88.5% of the colonies formed on YEMA medium plates were wet and gummy. Majority of the isolates did not grow on acidic pH (6 or below). PCR of 16S rDNA and sequencing led to identification of 25 representative isolates. A dendrogram developed based on partial 16S rDNA sequences delineated not only different genera of isolated rhizobia but also different species/strains indicating diverse nature of root nodule bacteria in hot arid soil environment. Genotypic and morphological associations were found to be independent of geographical affiliations.

Introduction

Nitrogen (N_2) is the most limiting factor for growth and biomass production in arid soils. Despite abundant supply of N_2 in the earth's atmosphere (~79%) as N_2 gas, it is unavailable for use by most organisms due to triple bond between the two nitrogen atoms, making the molecule almost inert (Cheng, 2008). Nitrogen to be used for growth must be "fixed" in the form of ammonium (NH_4) or nitrate (NO_3) ions. The conversion of N_2 into ammonia, and then into proteins, is achieved by specific bacteria through biological nitrogen fixation (Ormeño-Orrillo, 2013). The most important N_2 - fixing bacteria (NFB) are *Rhizobium*, belonging to family Rhizobiaceae that form symbioses with legumes (Figueiredo *et al.*, 2013). In India approximately 53.4% land area comprises of hot arid and semi-arid regions and arid western Rajasthan covers 61% of total hot arid area of the country (Tewari *et al.*, 2014; RajRAS, 2017). NFB face the challenges of high temperature, salt, osmotic stress, soil alkalinity and heavy metals in the hot desert soils (Rewald *et al.*, 2012). Therefore, more stress tolerant and efficient N_2 –fixing strains are required for arid environment in the soil. The information about diversity in natural

populations of NFB of arid ecosystem is necessary before the selection and application of the tolerant strains for biological N_2 fixation. However, the diversity of NFB is poorly described (Bahulikar *et al.,* 2014; Boakye *et al.,* 2016) and many of these microorganisms are yet undiscovered (Ondieki *et al.,* 2011). Assessment of diversity within native rhizobial population in different regions of the world has been receiving increasing attention (Latif *et al.,* 2013; Koskey *et al.,* 2018). The use of highly sensitive and accurate molecular methods has enabled the detection of large rhizobial diversity, with differentiation of closely related bacterial strains (Pancholy *et al.,* 2011; Boakye *et al.,* 2016; González *et al.,* 2019). Still there is inadequate knowledge of the diversity of rhizobia that nodulate important tree and crop legumes of the hot Indian arid zone (Choudhary, *et al.,* 2018). *Acacia senegal* (L.) Willd. and *Prosopis cineraria* (L.) Druce are two dominant multipurpose legume tree species of arid zone of India. The gum obtained from *A. senegal* is used in confectionaries and pharmaceutical industries (Pareek *et al.,* 2017); pods are relished by sheep and goat; and boiled and dehydrated seeds are consumed by local people as vegetables. *P.cineraria* or "khejri" is the backbone of rural economy of western Rajasthan as it provides fodder, fuel, vegetable etc and improves grain yield and forage biomass production of the associated field crops (Keerthika *et al.,* 2015; Bakhoum *et al.,* 2018). Clusterbean *or guar (Cyamopsis tetragonoloba L.)* and moth bean *(Vigna aconitifolia* (jacq.) Marechal) are two drought-hardy legume crops, commonly grown in arid and semi-arid regions of India. Clusterbean is reckoned with for export value of the "guar gum" extracted from the endosperm (Ramachandra *et al.,* 2015) and green pods of clusterbean, in the early stage, are used as delicious vegetable (Bhatt *et al.,* 2015). Moth bean is known for its tolerance to high temperature and soil moisture deficit situations (Sadashivanagowda *et al.,* 2017). Besides, as a source of pasture, fodder and green manure, it is also used in a variety of local dishes and snacks. There is little understanding of the diversity of indigenous rhizobial population accessible to these crops in the rhizosphere, a major requirement for enhancing symbiotic effectiveness between the legume host and the bacteria. Therefore, the aim of the present study was to explore the phenotypic diversity by studying the growth pattern and genotypic diversity by sequence comparison of highly conserved 16S rDNA molecules of the rhizobia that nodulate *A. senegal*, *P. cineraria*, cluster bean and moth bean growing in arid western Rajasthan.

Materials and Methods

Isolation of rhizobia

The root nodule samples were collected from 6-12 months old seedlings of *A. senegal* and *P. cineraria* growing in forest nurseries located in Jodhpur and Pali districts. Similarly root nodules were collected from guar and moth bean plants growing at farmers' fields from four districts of western Rajasthan viz., Jodhpur (26°15'26.5"N 72°59'39.9"E), Pali (24°45'N 75°50'E), Bikaner (28°03.983N 73°19.583E) and Barmer (25° 44'53.1" N 71°06'45.0" E) each representing different rainfall zones. For isolation of rhizobia, the roots bearing nodules were washed thoroughly with tap water and the nodules attached to a small piece of root were separated from the root system. The nodules were then surface sterilized with 70% alcohol for 3 min followed by 0.1% mercuric chloride for another 3 min and rinsed thoroughly with five washes of sterile, distilled water (Setu *et al.,* 2019). Each healthy nodule was crushed in a drop of sterile, distilled water in a sterile petri dish with the help of a sterile rod ((Muthini *et al.,* 2014). Loop-full of the

whitish suspension was then streaked on to yeast extract mannitol agar (YEMA; 31.83g L^{-1}; HiMedia Laboratories) medium plates having congo red dye (0.00125 mg/kg) and incubated at 28°C for seven days. *Rhizobium* like colonies that did not take up the red color of the dye were selected and single, isolated colonies were again streaked on to YEMA plates to get pure culture.

Culture maintenance

All rhizobial isolates were maintained on YEMA medium slants in test tubes containing congored dye and stored at 4°C until used. The isolates were restreaked on to YEMA medium plates for single colony isolation and checked for contamination every three months.

Morphological, Physiological and metabolic properties of rhizobia

Growth rate or the time taken for the isolates to form colonies on YEMA plates at 28°C was followed for the maximum period of seven days. Isolates were classified as fast and slow growers depending on number of days taken for the colonies to take full form. Colony morphology was scored as dry where the surface was smooth and firm, and wet where the surface was watery or slimy. Rhizobial isolates were screened for K solubilization on Aleksandrov agar medium having: 0.5% glucose, 0.05% magnesium sulfate heptahydrate, 0.0005% iron chloride, 0.01% calcium carbonate, 0.2% calcium phosphate, 0.2% feldspar (potassium aluminum silicate) and 1.8% agar (Prajapati and Modi, 2012).

The cultures were spot inoculated and incubated for 4-5 days at 28°C and looked for formation of a clear zone around the colony as a result of K solubilization. Screening for P solubilizing activity was done on Pikovskayas agar medium (HiMedia) modified by Sundara Rao and Sinha (1963) for detection of phosphate-solubilizing rhizobia. The cultures were spot inoculated and incubated for 4-5 days at 28°C. A clear zone around the colony was indicative of P solubilization by the isolate. The isolates were tested for their ability to grow on YEMA medium plates having pH range (4 to 8) adjusted by addition of acid or alkali during the preparation of the medium.

DNA extraction

Each rhizobial culture was inoculated separately in 150 ml conical flask containing 50 ml of autoclaved Luria broth culture medium and kept on to rotary shaker for 48 to 72h. The bacterial genomic DNA was isolated using HiPurA™ Bacterial and Yeast Genomic DNA purification spin kit of HiMedia as per manufacturer's instructions.

PCR amplification of 16S rRNA gene

The genomic DNA of each rhizobial strain was used for amplification and sequencing of 16S rRNA gene employing universal rDNA primers specific for conserved bacterial 16S rDNA sequences from *Eubacteria*- Eub1-forward (5'AGAGTTTGATCCTGGCTCA 3') and Eub2- reverse (5'GCTCGTTGC GGGACTTATCC 3'). Amplification was performed in a 50 µl reaction mixture containing: 1U Taq DNA polymerase (Bangalore Genei), 2.5 mM $MgCl_2$, 160 µM dNTP mix (Bangalore Genei), 50 pmol each of Eub1 and Eub2 primers, 50 ng genomic DNA in dH_2O. The reactions were performed in a thermal cycler (Corbett Research, USA) with following conditions: Initial denaturation at 95°C for 3 min followed by 36 cycles of one min. denaturation at 95°C, 30 s annealing at 50°C, 1 min 20 s elongations at 72°C with a final elongation step of 72°C for 10 min.

Gel electrophoresis and sequencing

Gel electrophoresis was performed with loading of 8 µl of 16S rDNA amplified product with 6x loading buffer (2 µl) on to 1.6 % agarose gel stained with ethidium bromide (10 mg/ml). 1 Kb DNA ladder (Biolabs) was used to estimate the band size. The gel was run in Tris-acetate-EDTA (0.5X TAE) buffer at 100V for 1h, visualized using a UV trans-illuminator and photographed under UV light using Syngene Gene Genius Bio Imaging system. The PCR products were directly sequenced using big dye terminator method in ABI prism DNA sequencer. Nucleotide sequence comparison was done with the bacterial 16S rDNA sequences available at database using the Basic Local Alignment Search Tool (BLAST) network services of the National Center for Biotechnology Information (NCBI), USA. Rhizobial cultures were identified based on the maximum similarity with the best aligned sequence of NCBI database. The Sequences were submitted to NCBI database and GenBank Accession numbers obtained.

Phylogenetic analysis

Phylogenetic analysis was done by multiple sequence alignment of the partial sequences of 16S rDNA molecules of 25 representative rhizobial strains using CLUSTAL X 2.0.11 software to determine the genetic relationships. Taxonomic position of each rhizobial culture was determined by construction of a phylogenetic tree depicting bootstrap values using NJ plot software (Perriere and Gouy, 1996).

Results and Discussion

Isolation of rhizobia

Out of 85 rhizobial cultures, 68 cultures were isolated from legume crops (35 from cluster bean and 33 from moth bean plants) and 17 were isolated from tree legumes (nine from *A. senegal* and eight from *P. cineraria*) growing in different districts of arid Rajasthan exhibiting different rainfall pattern (Table 1).

Morphological, Physiological and metabolic properties of the isolated rhizobia

All 85 rhizobial cultures were grouped into i) crop legume rhizobia (68) and ii) tree legume rhizobia (17) for the purpose of studying morphological, physiological and metabolic properties. Majority (58 %) of the isolated crop legume rhizobia were fast growers as the colonies were formed within 72 hrs, three percent were very fast growers where in colonies were formed in 24 hrs, 37% were slow growers with appearance of colonies after 72 hrs on YEMA plates at 28°C and 2% were very slow growing with colonies appearing after 5-7 days of incubation. In tree legume rhizobia, 52.9 % were fast grower, 35.3% were slow grower and 5.9% each were very fast and very slow growers (Table 2). As far as colony morphology is concerned, in crop legume rhizobia, majority (68.5%) of the colonies were wet, gummy and compact (WGC), 20% were wet, gummy and soft (WGS) as the colonies were merging with each other on slight tilting of the plate. In remaining 11.5%, the colonies were dry where the surface was smooth and firm. In tree legume rhizobia, 73% were wet, gummy and compact, 21% were wet, gummy and soft and remaining 6% were dry in colony morphology (Table 2).

Rhizobial isolates also varied in terms of their ability to grow in media at different pH levels. All the rhizobial isolates could not grow in media with lower pH levels (3.0, 4.0, and 5.0). At a pH level 6.0, 80% colonies showed restricted growth (barely managed to appear on the media plate), rest 20% showed normal

growth. All the isolates showed luxuriant growth at higher pH levels (7.0 and 8.0). None of the rhizobial isolates solubilized K or P.

Molecular characterization of rhizobial isolates

The diversity and relationships among the 25 representative rhizobial isolates were assessed using molecular methods. PCR amplification of 16S rDNA of the rhizobial isolates produced DNA bands of approximately 1100 bp size (Fig. 1).

Direct sequencing of each PCR product followed by sequence comparison against those available in the 16S rDNA database of NCBI led to identification of each bacteria up to species level. List of strains used for molecular study, their hosts, identification and their GenBank Accession numbers are given in Table 3. Most of the rhizobia isolated from moth bean were identified as *Sinorhizobium saheli*, except MBK-15 which was *S. chiapanecum*. Out of six cluster bean cultures, two were *Rhizobium pusense*, three were *Phyllobacterium leguminum* and one was *S. saheli*. All rhizobial cultures isolated from *A. senegal* root nodules were *S. saheli*. All *P. cineraria* rhizobia were also *S. saheli* except PC-14 which was identified as *S. kostiense* (Table 3).

Table.1 Isolation of rhizobial cultures

District	Rainfall (mm) range	Number of isolates			
		Cluster bean	Moth bean	*A. senegal*	*P. cineraria*
Pali	400-500	4	-	6	3
Jodhpur	300-400	4	3	3	5
NBPGR, RRS, Jodhpur	300-400	3	2	-	-
Bikaner	200-300	11	19	-	-
Barmer	200-300	13	9	-	-
Total	-	35	33	9	8

Table.2 Morphological, physiological and metabolic properties of rhizobia

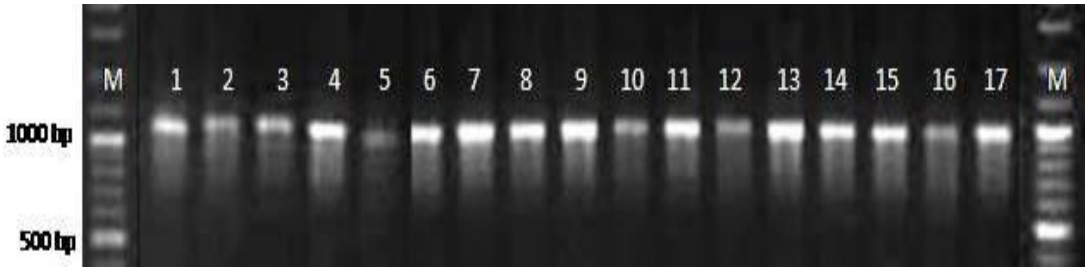
Percent rhizobia with different growth rates on YEMA medium				
Host	fast	Very Fast	Slow	Very slow
Crop legume	57.4	2.9	36.8	2.9
Tree legume	52.9	5.9	35.3	5.9
Percent rhizobia with different colony morphologies on YEMA medium				
Host	**Wet, gummy & compact**	**Wet, gummy & soft**	**Dry**	
Crop legume	68.5	20	11.5	
Tree legume	73	-	27	
percent rhizobia growing at different pH levels				
Host	**pH 3 to 5**	**pH 6**	**pH 7**	**pH 8**
Crop legume	No growth	Restricted growth-80%	Normal growth-100%	Normal growth-100%

		Normal growth-20%		
Tree legume	No growth	Restricted growth-100%	Normal growth-100%	Normal growth-100%
Percent rhizobia capable of solubilizing K and P				
Host	**Solubilization of K**		**Solubilization of P**	
Crop legume	Nil		Nil	
Tree legume	Nil		Nil	

Table.3 Identification, GenBank Accession nos. and place of isolation of rhizobial cultures

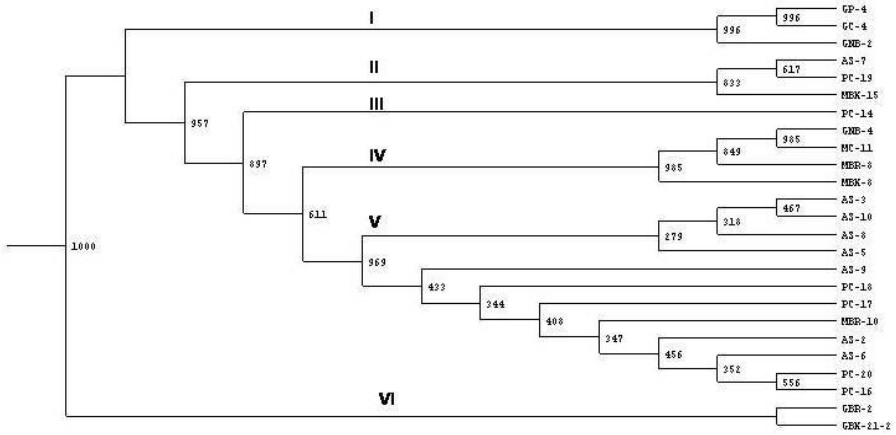
Strain	Host	Molecular Identification	GenBank Accession no.	District
MBR-8	Moth bean	*Sinorhizobium. saheli*	MF576133	Barmer
MBR-10	Moth bean	*S. saheli*	MF576134	Barmer
MBK-8	Moth bean	*S. saheli*	MF576135	Bikaner
MBK-15	Moth bean	*S. chiapanecum*	MF576136	Bikaner
MC-11	Moth bean	*S. saheli*	KY393040	CAZRI, Jodhpur
GBR-2	Cluster bean	*Rhizobium pusense*	MF576137	Barmer
GBK-21-2	Cluster bean	*R. pusense*	MF576139	Bikaner
GNB-2	Cluster bean	*Phyllobacterium leguminum*	KY393038	NBPGR, Jodhpur
GNB-4	Cluster bean	*Sinorhizobium saheli*	KY393039	NBPGR, Jodhpur
GC-4	Cluster bean	*Phyllobacterium leguminum*	KX101068	CAZRI, Jodhpur
GP-4	Cluster bean	*Phyllobacterium leguminum*	KX101069	Pali
AS-2	*Acacia senegal*	*Sinorhizobium saheli*	HQ738494	Pali
AS-3	*A. senegal*	*S. saheli*	HQ738495	Pali
AS-5	*A. senegal*	*S. saheli*	HQ738497	Jodhpur
AS-6	*A. senegal*	*S. saheli*	HQ738498	Pali
AS-7	*A. senegal*	*S. saheli*	HQ738499	Pali
AS-8	*A. senegal*	*S. saheli*	HQ738500	Jodhpur
AS-9	*A. senegal*	*S. saheli*	HQ738501	Pali
AS-10	*A. senegal*	*S. saheli*	HQ738502	Pali
PC-14	*Prosopis cineraria*	*S. kostiense*	HQ738506	Pali
PC-16	*P. cineraria*	*S. saheli*	HQ738508	Jodhpur
PC-17	*P. cineraria*	*S. saheli*	HQ738509	Jodhpur
PC-18	*P. cineraria*	*S. saheli*	HQ738510	Pali
PC-19	*P.cineraria*	*S. saheli*	HQ738511	Pali
PC-20	*P. cineraria*	*S. saheli*	HQ738512	Jodhpur

Fig.1 16S rDNA PCR product profile of 17 rhizobial cultures



M (Marker); 1(MBR-8); 2(MBR-10); 3(MBK-8); 4(MBK-15); 5(GBR-2); 6(GNB-2); 7(GNB-4); 8(GC-4); 9(AS-2); 10(AS-3); 11(AS-5); 12(AS-6); 13(AS-7); 14(PC-14); 15(PC-16); 16(PC-17); 17(PC-18)

Fig.2 Dendrogram of 25 rhizobial isolates based on amplified 16S rDNA partial sequence data



A dendrogram was generated by comparing 16S rDNA partial sequences of all the 25 rhizobial cultures isolated from two crops and two tree legumes with bootstrap values mentioned against each delineated cluster (Fig. 2). Based on the dendrogram, rhizobial cultures can be distinctly divided into two clusters or groups- A and B. Group A is further divided into five subgroups (I to V), and group B consists of only one subgroup (VI). Within Group A, Subgroup I, consists of three clusterbean isolates, GP-4, GC-4 and GNB-2 isolated from Pali (400-500 mm rain fall), CAZRI Research farm, Jodhpur and NBPGR Research farm, Jodhpur (300-400 mm rain fall) respectively. Subgroup II consisted of three isolates AS-7 (from *A. senegal*), PC-19 (from *P. cineraria*) both isolated from Pali and MBK-15 a moth bean culture isolated from Bikaner (200-300 mm rain fall). Subgroup III consisted of only PC-14 (from *P. cineraria*) isolated from Pali. Subgroup IV consisted of four isolates, GNB-4 isolated from cluster bean growing at NBPGR Research farm, Jodhpur, MC-11, MBR-8 and MBK-8 isolated from moth bean plants growing at CAZRI Research farm, Jodhpur, Barmer (200-300 mm rain fall) and Bikaner fields respectively. Subgroup V was the largest consisting of 12 rhizobial isolates- AS-2, AS-3, AS-6, AS-9 and AS-10 isolated from *A. senegal* seedlings collected from Pali nursery and AS-5 and AS-8 isolated from *A. senegal* seedlings collected from Jodhpur nursery. Subgroup V also consisted of isolates PC-16, PC-17, PC-18, PC-20 isolated from *P. cineraria* seedlings collected from Jodhpur and Pali nurseries. One moth bean isolate MBR-10 was also clustered within subgroup V, which was isolated from Barmer.

Subgroup VI from Group B consisted of two guar cultures GBR-2 and GBR-21-2, both isolated from Barmer, made a distinct cluster from rest of the isolates (Fig. 2).

Out of 85 rhizobial cultures isolated in the present study, 68 were from crop and 17 were from tree legumes. Rhizobia were found to be diverse in their growth rate, colony morphology as well as their response to different levels of pH in the growth medium irrespective of the host as well as their agro-climatic zones of isolation. The rhizobial isolates that formed colonies on YEMA media within three days of incubation were classified as fast growers based on the work of Odee *et al.,* (1997). Both fast and slow growers were found to exist in the tropical rhizospheric soils of the four districts where the host legumes were growing. This finding concurs with earlier studies of Ondieki *et al.,* (2017) that reported the appearance of both fast- and slow-growing rhizobia from arid and semiarid lands of lower eastern Kenya but rapidly growing isolates were more common in the three soil sampling sites and fast growers formed majority of the nodules. In our study also, fast growing rhizobial cultures were found in higher numbers as compared to the slow growing ones in both crop as well as tree legume isolates indicating that fast growers outperform slow growers as far as nodule forming capability is concerned. Sanginga *et al.,* (1989) also reported in tropical soils, that although *L. leucocephala* was nodulated by both fast and slow-growing rhizobia, effective nitrogen fixing nodules were formed with the fast-growing rhizobia only. Rhizobia from low rainfall regions have the ability to multiply fast within short rains and are more tolerant to stress conditions than slow-growing strains as a survival strategy in challenges environmental conditions. We encountered three forms of colony morphologies in the crop legume rhizobia - wet, gummy and compact (WGC)-68.5%; wet, gummy and soft (WGS)-20% and dry form (D)-5% where as in tree legume rhizobia we found WGC-73% and D-27% forms only. The production of exopolysaccharides (EPS) to varying levels points towards the versatility and survivability of the rhizobial isolates to withstand various physiological stresses present in the arid/semiarid climate as explained by Karthik *et al.,* (2017). Under present study, in all cases the rhizobial growth was normal when pH of the medium was alkaline (7 or 8). This may be because the pH of soils of the regions where the host legumes were growing is mostly alkaline and rhizobia isolated from such soils were adapted to the alkaline conditions (Sankhla *et al.,* 2018). Fast-growing rhizobia are generally considered to be less tolerant of acidic conditions than slow-growing strains. Indeed, soil acidity alone is responsible for significant losses in global legume production, resulting from impaired plant and rhizobial associations, in addition to decreased nodule development and nitrogen fixation (Ferguson *et al.,* 2013). However, Boakye *et al.,* (2016) working on characterization and diversity of tree legume rhizobia growing on three different types of soils having acidic pH in Ghana reported that most of the tree legume rhizobia were fast-growers and acid tolerant.

For molecular diversity study, 16S rDNA is most suitable to genetically group strains of rhizobia since it constitutes the basis of rhizobial classification and identification of new and novel forms (Kuykendall, 2005; Yang *et al.,* 2016). Amplification of the 16S rRNA gene of rhizobial isolates used in this study resulted in a single band of approximately 1.1 kb size. Slight variation in the sizes of the PCR products as evident from the 16S rDNA PCR profile was indicative of diversity in the rhizobial cultures. There is predominance of *Sinorhizobium* (*Ensifer*) strains in our study indicating that *Sinorhizobium* species are more adapted to

alkaline soils and arid conditions of Thar Desert and are dominant microsymbionts of native legumes in the Thar Desert (Rathi *et al.,* 2017). Construction of a dendrogram based on partial sequences of the 16S rRNA gene divided the 25 rhizobial isolates into two distinct clusters or groups- A and B, which were further subdivided into six subgroups indicating diversity both at intra- and inter-specific levels. Most of the *P cineraria* and *A. senegal* isolates (11 out of 14) were clustered together in one clade or monophyletic subgroup (V) and all were *S. saheli* indicating that these rhizobia have host range restricted to tree species and therefore could be used as an inoculum in forestry. Host range restriction also seems to be the case for both guar rhizobia (sub group I) and moth bean rhizobia (sub group IV) although some isolates seemed to be closer to those isolated from *P cineraria* or *A. senegal* in other subgroups (II and V) and can be useful in preparation of consortia for extension of agricultural practices (Tak and Gehlot, 2019). Dendrogram was also able to delineate PC-14 (*S. kostiense*) into a separate subgroup III and two *R. pusense* cultures in to sub group VI.

Our findings indicate that the soils of areas under study harbor populations of highly diverse rhizobial strains well adapted to local climatic and edaphic factors. This finding is also in agreement with the results obtained by Boakye *et al.,* (2016) based on their studies with diverse *Rhizobium* cultures isolated from tree legumes growing in nutrient deficient soils of Ghana. Findings of Dekak *et al.,* (2018) with rhizobia nodulating wild legumes *Genista microcephala* and *Argyrolobium uniflorum* in northeastern Algeria and Choudhary *et al.,* (2018) with *Ensifer* strains nodulating *Senegalia* (*Acacia*) *senegal* growing in arid regions of Western Rajasthan, India also support our study. This also provides ample opportunity to select effective, indigenous stains for bioinoculant production to enhance growth of native crops and trees in mostly N-depleted soils of the hot deserts of India.

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